ZOOLOGICAL SCIENCE 9: 1081–1085 (1992)

[COMMUNICATION]

Sexual Development of Immunocompetence in the Toad, *Bufo regularis*

Abdel Hakim Saad

Zoology Department, Faculty of Science, Cairo University, Cairo 12613, Egypt

ABSTRACT—The kinetics of primary anti-rat erythrocytes (RRBC) antibdody were compared in juvenile and adult toads *Bufo regularis*. The antibody titers were measured by haemagglutination assay, and antigen specific rosette-forming cells were enumerated in the spleen using immunocyto- adherence assay. Both adults and juveniles responded to RRBC, with adult being always the highest responder. Among the adult toads, slight differences between male and female could be observed. However, juvenile toads did not demonstrate sex-associated immune differences.

INTRODUCTION

Immunocompetence is a complex physiological process that develops during ontogeny and is maintained throughout animals's life cycle. Anuran amphibians are excellent models for immunologic studies from the perspectives of both evolutionary and developmental biology [1, 2]. In comparison with amniotes, there is an obvious advantage in using the amphibian models for studying the ontogeny of immune responses, since amphibians develop as free-living embryos and larvae thus allowing experimental manipulation from the earliest stages of immune system development [3, 4]. However, little attention was paid to the immune response of anuran amphibians during sexual maturity and ageing [5, 6]. Here, we examine the importance of major biological variables such as sex and age on the humoral immune response of the toad, *Bufo regularis*. In addition, the role of sex hormones played in the immune response of amphibians is discussed.

Accepted July 4, 1992
Received January 7, 1992

MATERIALS AND METHODS

Animals and housing Male and female toads, *Bufo regularis* (Anura, Bufonidae), were obtained from Abu Rawash area in the vicinity of Cairo (Egypt). Toads were kept in glass aquaria with tap water in a sunny animal room at ambient temperature of 20–27°C during spring and provided with granulated trout feed *ad libitum*.

Cell preparation Individual spleens were excised carefully and gently teased apart with fine forceps over stainless-steel meshes in ice-cold, serum-free Leibovitz (L-15) medium (CIBCO, Grand Island, N.Y., USA). Tissue debris were decanted, and cells were washed twice with ice-cold medium by centrifugation ($400 \times g$, 5 min). In the last wash, pellets were counted and their viability was assessed by the trypan blue exclusion test.

Histological procedure After dissection, the spleen was fixed in 10% buffered formalin, dehydrated in alcohol and embedded in paraffin. Eight μm-thick sections were stained with haematoxylin and eosin.

Antigen and immunization Rat erythrocytes (RRBC) were obtained from at least three animals. Pooled heparinized blood was centrifuged

(400×*g*, 5 min) and the cells were washed thrice with phosphate-buffered saline (PBS), pH 7.2 before use. Preliminary experiments disclosed that 0.4 ml of 10% RRBC is an optimum dosage which induced maximum antibody (Ab) response when given intraperitoneally (i.p.). Control unimmunized toads were injected i.p. with 0.4 ml PBS, pH 7.2. Immunized and control blood samples were allowed to clot for 2 hr at room temperature and then overnight at 4°C. After centrifugation (650× *g*, 15 min), individual sera were heat-inactivated at 56°C for 30 min and stored at −20°C until use.

Assay of humoral immune response Groups of animals were decapitated at specific intervals after immunization and a single spleen cell suspension was prepared as described previously. Antigen specific rosette-forming cells (RFC) were enumerated in the spleen using immunocyto-adherence assay as described by Saad and Ali [7]. On the basis of actual number of cells for each sample, the RFC per 10^6 spleen cells was computed. Serum Ab titers were determined in standard haemagglutination assay as described previously [7]. Ab titers were expresed as $\log_2$ reciprocal of the highest dilution showing microscopic agglutination.

Statistical analysis Student t-test was used to determine levels of significance between control and experimental groups. Diferences were considered to be significant at P values <0.05.

RESULTS AND DISCUSSION

Field studies have shown that the growth rate of anuran amphibians, as determined from snout-vent (SV) length, can be used as an indicator of growth and sexual maturity [8, 9]. As depicted in Figure 1, the total number of splenic lymphocytes recovered from males and females appear to increase with growth. At SV-length greater than 95 mm, the lymphocyte population starts to decline, possibly reflecting an increase in animal age and approaching senescence. However, no sex-related differences were observed between male and female toads. For simplicity, we analyzed comparatively the variations in the kinetics of immune response between two different SV-length: 30–55 mm (i.e. juvenile toads) and 80–95 mm (i.e. adult toads). The main issue raised in our study is that the response to RRBC in *B. regularis* appeared to be highly age-dependent but not sex-dependent. Juvenile animals showed a lower RFC and Ab titers. A considerable number of RFC were detected in male and female toads on the first four days after RRBC injection. Thereafter, a sharp decline occurred in the number of RFC during the subsequent periods. The shape of the curves was otherwise quite similar (Fig. 2A). However, despite the fact that the peak of RFC response to RRBC was somewhat delayed and diminished in adult males in comparison with adult females, yet sex-associated differences at some time point were not significant (Fig. 2B). The kinetics of the primary anti-RRBC Ab response are depicted in Figure 2. It can be seen that, in adult toads, primary response appeared to be more vigorous, and total Ab titers were significantly higher ($P<0.001$) than those of juvenile toads (Fig. 3A). Also, with adult female toads the peak of primary Ab response to RRBC is somewhat delayed in comparison with adult male toads (Fig. 3B).

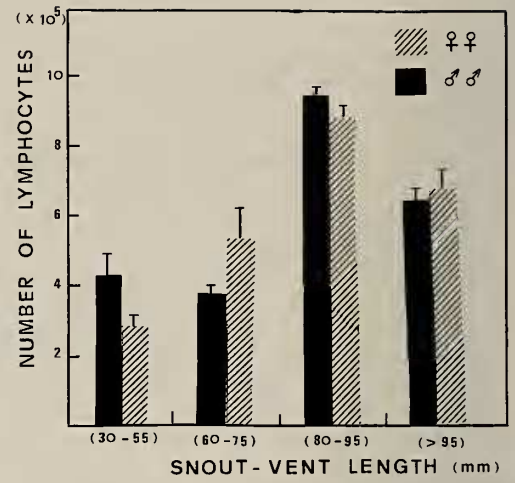

Fig. 1. Total number of viable splenic lymphocytes obtained from female and male toads, *B. regularis* of varying snout-vent length. Each column represents the mean number of 10–15 separate animals. Vertical bars indicate standards error of the mean.

It is concluded that juvenile toads have the competence to respond to RRBC but their humor-

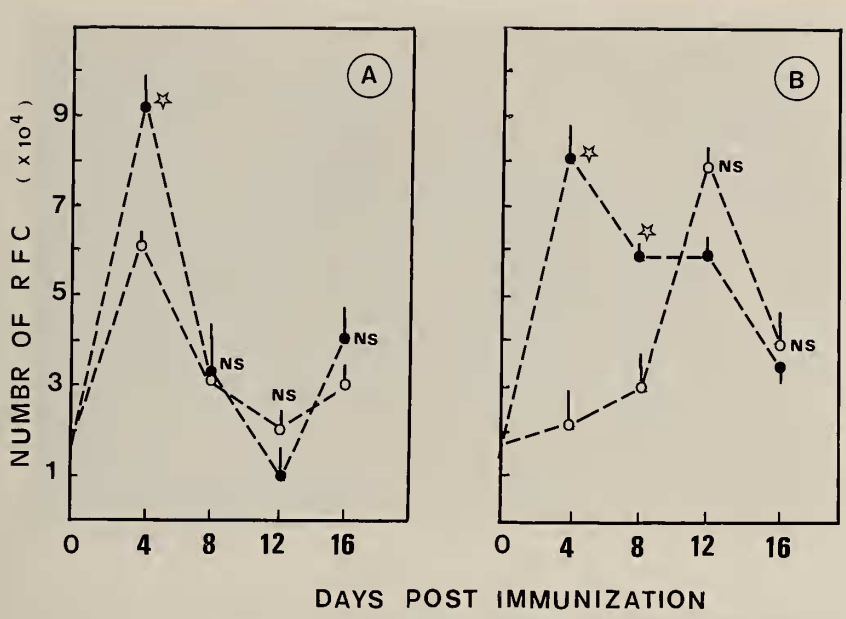

FIG. 2. Kinetics of rosette-forming cells (RFC) response in the spleen of female (●—●) and male (○—○) toads, *B. regularis*. Juvenile (A) and adult (B) toads were immunized on day O with 0.4 ml 10% RRBC suspension. Each point represents the mean response of 3–5 separate animals and the vertical bars indicate standard error of the mean. ☆=$0.05<P<0.01$ and NS=not significant.

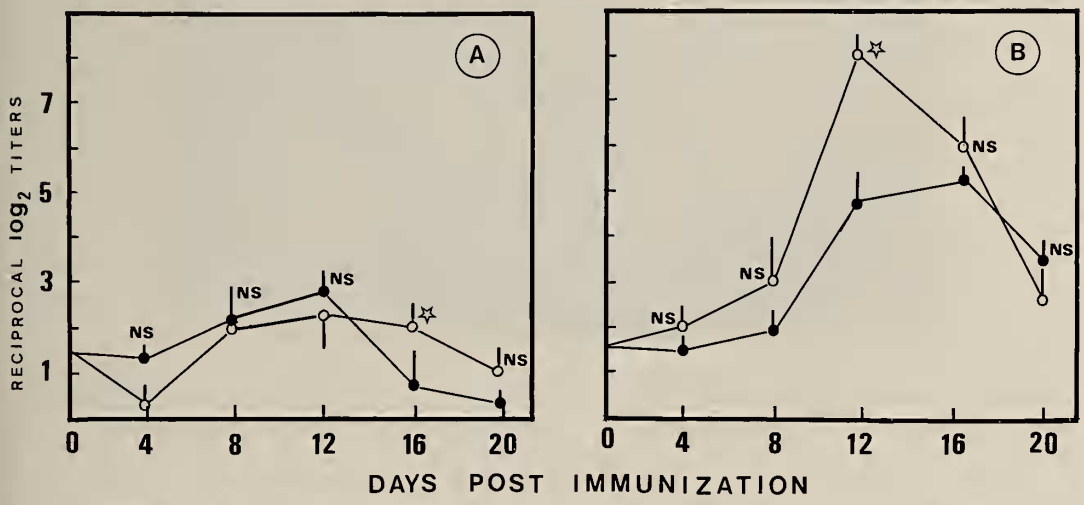

FIG. 3. Mean serum haemagglutinin titers in female (●—●) and male (○—○) toads, *B. regularis*. Juvenile (A) and adult (B) toads were immunized on day O with 0.4 ml 10% RRBC suspension. Each point represents the mean response of 3–5 separate animals and the vertical bars indicate standard error of the mean. ☆=$0.05<P<0.01$ and NS=not significant.

al immune system is still not full grown. Taken together, these observations present the possibility that the maturation of the immune response to RRBC depends on the maturational status of the spleen, which in juvenile toads has a poorly defined histology, relatively low number of lym-

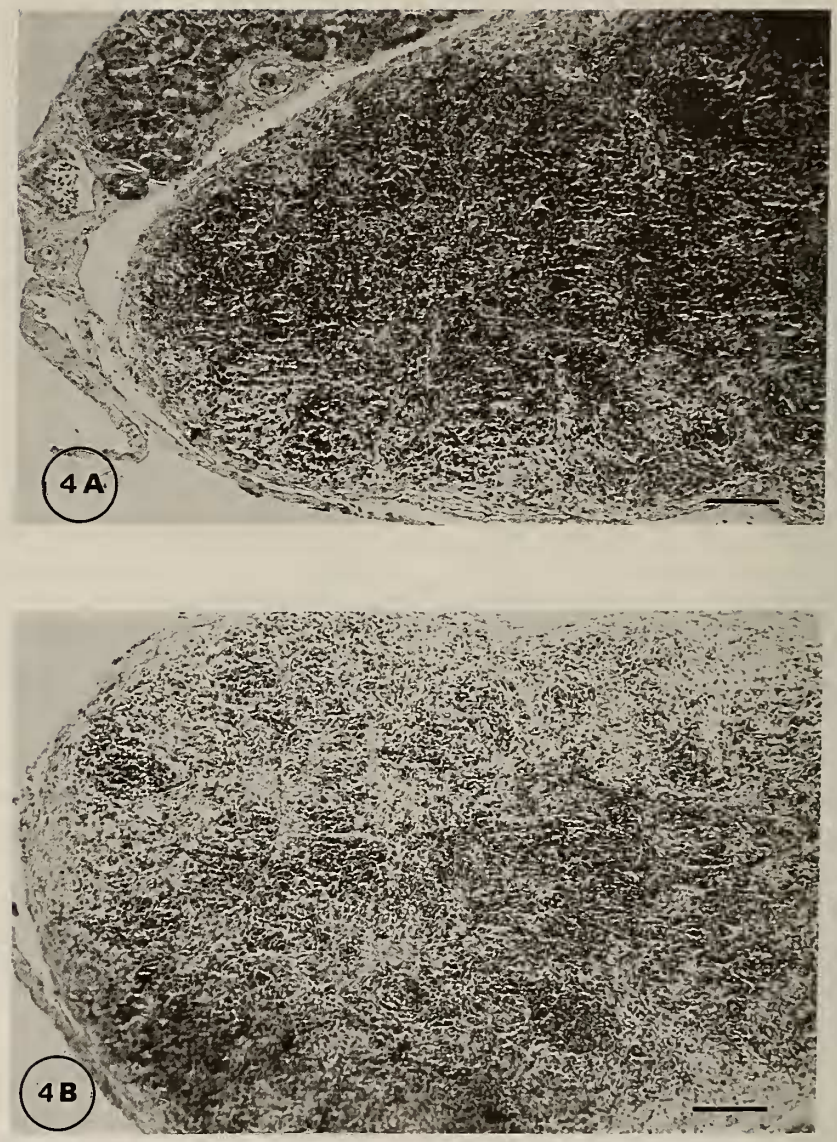

Fig. 4. The spleen of *B. regularis* sacrificed during mid-spring. (A) Spleen of adult toads with the thickened wall of the central arteriole which is densely populated with lymphoid cells. (B) Spleen of juvenile toads with a considerable number of lymphoid cells which are present in the red and white pulp. Scale bar: 100 μm.

phoid cells, and small mass. Histologically, the spleens were large in adult toads, and their white pulp was filled with numerous lymphocytes aggregated around the blood vessels (Fig. 4A). In contrast, the spleens were smaller and significantly lighter in juvenile toads and the lymphocyte aggregations were diminished (Fig. 4B). Immunological maturity, therefore, attained only when the pool of immunocompetent cells in the spleen becomes sufficient in size and diversity.

The data reported here, which suggest the assumption of sexual maturity may relate to greater reactivity to RRBC, are also of interest when considering hormonal systemic controls on immune regulation. The shift in immune reactivity with sexual maturity in *B. regularis* should be

viewed in the context of the data which suggest constancy at a lower reactivity level in sexually immature juveniles [5, 6]. In marine teleosts, the humoral activity of mature females to sheep erythrocytes (SRBC) was lower than males or immature females in the spawning season [10]. Response to SPRC in partridges was found to be sex-independent, but declined markedly in partridges older than 1-years [11]. Other studies on pigeons have demonstrated that maturation of the immune functions in terms of anti-SRBC rosette formation and migration inhibition kinetics occurs at 3–4 months of age, which coincides with the onset of sexual maturation [12]. Therefore, although the evidence is circumstantial, the changes found in the activity of *B. regularis* to RRBC could be imputed to the influence of sex hormones. It is clear from our study that during spring, i.e. the time of sexual maturation in amphibians of temperate regions [13, 14], the immunologic responses investigated are different between juvenile and adult animals. Indeed, before sexual maturation, the humoral responses are similar between the sexes. It seems that the onset of the reproductive maturity triggers some differential capacity in the immune reactivity between juvenile and adult toads. This observation alone unequivocally supports the role of sex hormones in the immune expression. However, the mechanisms by which sex hormones control the immune response are not known. It is also not known whether testosterone and estradiol have opposing effects on the same cells or whether each hormone acts on different arms of the regulatory circuits of the immunologic network [15, 16].

REFERENCES

1 Du Pasquier, L. (1976) In "Comparative Immunology", Ed. by J. J. Marchalonis, Blackwell Scientific Publ., Oxford, pp. 390–419.

2 Katagiri, C. and Tochinai, S. (1987) Develop. Growth and Differ., **29**: 297–305.

3 Flajnik, M. F., Hsu, E., Kaufman, J. F. and Du Pasquier, L. (1987) Immunology Today, **8**: 58–64.

4 Ruben, L. N., Clotheir, R. H., Horton, J. D. and Balls, M. (1989) Bio Assays, **10**: 7–12.

5 Wright, R. K. and Cooper, E. L. (1980) In "Development and Differentiation of Vertebrate Lymphocytes" Ed. by J. D. Horton, Elseriver, North Holland, pp. 141–152.

6 Ruben, L. N., Clotheir, R. H., James, H. S. and Balls, M. (1984) Cell Differentiation, **14**: 1–5.

7 Saad, A.-H. and Ali, W. (1992) Zool. Sci., **9**: 349–356.

8 Ryan, R. A. (1953) Copeia, **1953**: 73–80.

9 Hagstorm, T. (1977) Zool. Scripta, **6**: 61–68.

10 Nakanishi, T. (1986) Vet. Immunol. Immunopathol., **12**: 336–342.

11 Randi, E., Chiricolo, M., Spagesi, M., Ghendini, I., Savigni, G., Giovannini, A. and Franchesi, C. (1985) Dev. Comp. Immunol., **9**: 679–690.

12 Selvaraj, P. and Pitchappan, R. M. (1988) Dev. Comp. Immunol., **12**: 379–384.

13 Lofts, B. (1984) In "Marchall's Physiology of Reproduction". Ed. by G. E. Lamming, Churchill Livingstone, Edinburgh, Vol. I, pp. 127–305.

14 Moore, E. L. and Zoeller, R. T (1990) Horm. Behav., **13**: 207.

15 Forsberg, J. G. (1984) Arch. Toxicol., **55**: 79–87.

16 Grossman, C. J. (1984) Endocrin. Rev., **5**: 435–455.